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16. ABSTRACT

A committee of scientists was established by the U.S. Environmental Protection Agency to appraise the current state of aneuploidy test methodology, to compile and analyze published data on the chemical induction of aneuploidy, and to provide guidance for additional test development and validation. The committee was organized into 9 working groups, representing different eukaryotic organisms ranging from fungi to whole mammals. The Environmental Mutagen, Carcinogen, and Teratogen Information Program at Oak Ridge National Laboratory identified and provided literature to the working groups. The 9 reports were prepared by the individual working groups. The reports include: (1) descriptions of the available testing approaches and general principles to be followed for protocol development, (2) criteria for evaluating data, and (3) recommendations for future test development and basic research. The evaluated results are presented in tables. For the positive and negative calls, the lowest effective dose tested or the highest ineffective dose tested, respectively, and the range of concentrations tested are provided. An overall summary report was prepared that summarizes the conclusions and recommendations of the 9 working groups and the chemical data base. It is hoped that these reports will provide a framework for further test development and validation of aneuploidy assays.

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Chemically induced aneuploidy in mammalian cells in culture *

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* Report of the Aneuploidy Data review Committee (Office of Research and Development, U.S. Environmental Protection Agency, Washington, DC).

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Summary

Our objectives were to assess whether there exist useful aneuploidy tests in vitro, to identify chemicals that showed potential for mitotic aneuploidy induction, and to recommend some features of suitable protocols for such testing. From over 100 papers we selected 24 for review. The acceptable studies examined hyperdiploidy at metaphase, had concurrent negative controls with low background rates of hyperdiploidy, used a fixation time sufficient for cells to complete more than one cell cycle after treatment and had multiple dose levels with at least 100 cells scored per point. We judged that 12 compounds were positive, 7 inconclusive, and 4 negative with the reservation that 2 of the 4 compounds had not been tested up to toxic doses. Many of the positive compounds are also known to cause structural chromosome aberrations. We separately reviewed qualitative reports of 'C-mitotic' effects, anaphase lagging, multipolar mitoses, or altered DNA content, since these effects may sometimes by associated with aneuploidy induction.

No well-validated in vitro aneuploidy assay exists, and much research is required to develop tests, perhaps using chromosome counts, DNA content, or effects on cell organelles necessary for mitosis. In test protocol development we should carefully consider choice of cell sample size, use of in vitro metabolic activation systems, and selection of doses, especially with regard to the problem of whether cytotoxic concentrations should be used.

(1) Introduction

Screening tests for chemicals are used widely to detect mutagens in vitro, for example by chromosome breakage, an end point thought to be linked with heritable genetic defects and with cancer induction. It is important to find out whether chemicals exist which might increase the burden of human aneuploidy but are not detectable in assays designed for screening other types of mutagens and carcinogens. We need to establish whether mitotic aneuploidy is a predictor of meiotic effects, but in addition to concern about effects on subsequent generations, chemicals that cause mitotic aneuploidy might also be treated with caution because of the association of aneuploidy and other chromosomal changes with cancer (e.g., Benedict et al., 1983; Sandberg, 1983; Rowley, 1983; Dryja et al., 1984).

In contrast with the review on cytogenetic tests prepared by the U.S. Environmental Protection Agency Gene-Tox committee (Preston et al., 1981), this report cannot define testing protocols, nor can we list many chemicals which clearly do or do not induce aneuploidy in cell cultures, because so few researchers have set out to identify these. In vitro aneuploidy tests involve treatment of cultured cells and chromosome counts at metaphase in subsequent cell generations. The literature contains no large historical collection of data or of descriptions of test systems.

For this report, aneuploidy was defined as induction of numerical changes involving whole chromosomes. Where chromosome counts were made on metaphase cells, only hyperdiploidy was accepted as an indication of aneuploidy induction. In rare cases where numerically abnormal clones were established and grown for several successive cell cycles, both hypo- and hyperdiploid clones were accepted as aneuploid. Since induction of polyploidy (i.e., cells with even multiples of the haploid number of chromosomes) may not result in aneuploidy, our definition of hyperdiploidy included cells with between $2N$ and $3N$, where N is the haploid number of chromosomes.

It should be emphasized that induction of aneuploid nuclei after *mitotic* division does not prove a compound's potential to induce aberrant

meiotic division. Because chromosome rearrangements can interfere with pairing at meiosis and lead to aneuploidy, compounds that break chromosomes may also induce aneuploidy at meiosis. However we excluded studies of mitotic structural aberrations because clastogens should be detected in chromosome breakage assays while the end point of interest in the present report was numerical abnormalities. Of the 103 papers reviewed, we accepted 24 and rejected 79 of which 18 were retained for consideration of mitotic abnormalities (see Selection criteria, Section 2).

(2) Selection criteria

Papers were not evaluated further if they fell into the general categories for rejection established by the work group (see Dellarco et al., 1985, this volume), or when:

(1) Data were on chromosomal structural aberrations only (metaphase or anaphase).

(2) Chemical treatment occurred in vivo, even if cells were subsequently studied in vitro.

(3) Germ cells were used as the test system.

(4) Conclusions were based on biochemical genetic studies that were thought to detect aneuploidy on principles similar to studies in organisms such as fungi. These methods did not prove whole-chromosome aneuploidy.

(5) An inappropriate end point was studied, such as somatic pairing or satellite associations.

The papers reviewed fell into 4 categories.

(1) Papers that set out to test for aneuploidy induction. The acceptable papers were those which reported metaphase numerical aberrations, in particular hyperdiploidy. Anaphase aberrations alone were not considered evidence for aneuploidy. They were used only if a clear increase in lagging objects was seen along with other observations of numerical changes, because among laggards we cannot distinguish fragments from whole chromosomes. Many authors described cell morphology and gave qualitative information only, for example describing a C-mitotic effect or abnormal mitoses. In formulating a set of criteria for acceptance or rejection of data, we decided to retain such data in a category of qualitative observations including polyploidy and C-mitosis. Multipolar mitoses were also in this category since they result in lethality rather than in aneuploidy (e.g., Teplitz et al., 1968).

(2) Papers that investigated mechanisms of aneuploidy induction in vitro.

(3) Papers aimed at developing test methods for aneuploidy.

(4) Studies of aneuploidy in association with other phenomena such as chemical transformation.

In categories 2, 3 and 4, numerical chromosome changes wee not usually the end point studied. The reports concerning chemical effects on cell organelles thought to be vital for chromosome movement and cell division, such as centrioles, kinetochores, and microtubules, did not usually give quantitative data and were used only for discussion. Similarly, when the end point was DNA content without confirmation by microscopic chromosome analyses, the study has been discussed separately, because of the problem of distinguishing clastogenic effects from numerical changes.

Papers remaining after this first review were then evaluated for suitability of the protocols used, and data were accepted only if the following criteria were met:

(1) A concurrent negative control was used.

(20 The background rate of aneuploidy was low, e.g. $\leqslant 5\%$.

(3) The fixation time was long enough after treatment to allow aneuploidy to appear; the first mitotic division after treatment was not suitable. This time depended on the cell line used: if the cell cycle length were 12 h a fixation time of 14 h, for example, might sample cells that were in the second metaphase after a G_2 treatment, but later times would be required to examine cells that were in S or G_1 at the time of treatment.

(4) At least two doses were tested. We planned to accept clearly positive results if only a single dose was tested, but no such examples arose in studies that were otherwise acceptable.

(5) Data on hyperdiploidy were shown clearly and not pooled with results on polyploidy and/or hypodiploidy.

(6) Sufficient cells were scored: 100 per dose. Exceptions were made for one or two studies with 50–75 cells per dose, but the results were classed as inconclusive.

Exogenous metabolic activation was not made a

requirement, because this was used so rarely. However, the use of activation systems or cells with metabolizing capacity should certainly be considered for future testing.

(3) Criteria for positive, negative, and inconclusive assessments

(3.1) Aneuploidy evaluation

Positive. A clear increase in hyperdiploidy was required. We did not use a strict requirement for statistical significance of the results, since widely accepted statistical methods have not been developed, but we judged the increase based on experience with structural chromosomal aberrations in vitro (see, for example, Archer, 1981). In some cases, distributions of chromosome numbers among cells were shown as a histogram and our judgement was based on a clearly visible shift in the frequency of hyperdiploid cells.

Negative. There were few clearly negative results. Ideally a negative conclusion would be drawn only if large numbers of cells were scored and there was evidence that the compound reached the cells, e.g., from cytotoxicity, reduced mitotic index, or structural aberrations. Of the 4 compounds judged negative in this review, two were qualified because in one case there was no information on toxicity, and in the other there was no apparent toxicity in terms of altered mitotic indices.

Inconclusive. Data were considered inconclusive if (a) there were too few cells scored; (b) if a weak increase did not achieve significance when tested by the authors or was not repeatable; or (c) if the increase was marked but found only for hypodiploidy, not hyperdiploidy.

(3.2) Clastogenicity evaluation

We were interested in a comparison of results of aneuploidy testing with induction of structural chromosomal aberrations in order to identify any compounds that appeared to induce numerical but not structural changes. We did not carry out a comprehensive review for all the compounds, but did make a literature search of the EMIC * data base for most of the compounds evaluated. Evidence for induction of aberrations in vitro and in vivo is summarized in Tables 3 and 4 with representative references. For two compounds, distamycin A and zarontin, no data on chromosome aberrations were obtained, but distamycin A affects chromosome condensation (Ronne et al., 1982). In assessing the aberration data we used criteria similar to those of Preston et al. (1981). We rejected abstracts, papers in foreign languages, and papers that showed conclusions without data. The results were considered inconclusive if there were few cells scored, a single dose reported, no fixation time given or an inappropriate fixation time used, no statistically significant increases, no dose relation, or if chromatid gaps were included in the total aberration frequencies such that the numbers of other aberrations could not be determined.

(4) Results

The aneuploidy results are presented in Tables 1 and 2 and summarized in Tables 3 and 4. Our final assessment (Table 1) covered 23 compounds. Of these, 12 were judged to be positive, 7 inconclusive, and 4 negative. (Two of these were qualified negatives because toxicity was not demonstrated in the test.) Of the positive compounds (Table 3), three are known spindle disruptors, colcemid, vinblastine and griseofulvin; also, diazepam had a C-mitotic effect (Tables 1 and 2), which suggests spindle disruption that might lead to aneuploidy. Three of the positive compounds are also DNA-binding mutagens and clastogens: benzo[*a*]pyrene (BaP); dimethylbenzanthracene (DMBA) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). A fourth mutagen, the alkylating agent mitomycin C, was found negative in a test for increased frequency of metaphase lymphocytes with an extra Y chromosome (Table 1) and needs further testing to establish whether it induces aneuploidy in other systems. The other compounds positive in aneuploidy tests were carbaryl, a carbamate pesticide; benomyl, an antihelminthic whose breakdown products include methyl benzimidazole carbamate; the hormone and carcinogen diethylstilbestrol (DES); the promoter 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and fibers of chrysotile asbestos.

* Environmental Mutagen, Carcinogen and Teratogen Information Department, Oak Ridge National Laboratory, TN.

TABLE 1

IN VITRO MAMMALIAN CELL ANEUPLOIDY

Chemical (CAS Registry No.)	Cells [a]	Tox [b]	Aneuploidy		Range tested [d] (μg/ml)	HIDT [e] (μg/ml)	LEDT [e] (μg/ml)	Reference
			+ / −	Type [c]				
Asbestos (chrysotile) 12001-29-5	SHE	+	+	hr, ho, pp	0.5–2.0 $\mu g/cm^2$		1 $\mu g/cm^2$	Oshimura et al., 1984
Benomyl/ benlate 17804-35-2	HPBL	n	+	hr:YY			1	Tenchini et al., 1983
	HPBL	?y	−	hr, ho		20000 ppm		Gupta and Legator, 1976
Benzo[*a*]pyrene 50-32-8	SHE	n	+	hr			10	Benedict et al., 1972
Benzanthracene 56-55-3	SHE	n	I [f]	hr	0.1–6			Benedict et al., 1972
Carbaryl (1-naphthyl-*N*-methyl carbamate) 63-25-2	V79	?	+	hr, pp			20.1	Onfelt and Klasterska, 1983
Colcemid 477-30-5	HPBL	? [g]	I [h]	hr:YY	0.03–0.3			Tenchini et al., 1983
	BHK21	?	+	hr, pp clones			0.07	Barass, 1982
	DON	?	+	hr, pp			0.05	Hsu et al., 1983b
	CHW	?	+	hr, pp clones			0.02	Cox, 1973
	V79, HH	?	+	hr, pp			0.02	Kopnin and Stavrovskaya, 1975
	DON	?	+	hr, pp, cm			0.05	Kato and Yoshida, 1970
	CHW	?	+	clones			0.02–0.03	Cox et al., 1976
	SHE	y	+	hr, ho, pp	0.01–1.0		0.03	Tsutsui et al., 1984
Cyproheptadine hydrochloride 969-33-5	HPBL	n [i]	−	ho, hr		69.4		Hite et al., 1977
Diazepam 439-14-5	DON	?	+	hr, mp, cm			100	Hsu et al., 1983a
Diethylstil-bestrol 56-53-1	SHE	n	+	ho, hr			1.0	Tsutsui et al., 1983
9,10-Dimethyl-1,2-benzanthracene 57-97-6	SHE	n	+	hr			0.05	Benedict et al., 1972
Distamycin A 636-47-5	HPBL	?	I [h, j]	hr:YY	25–100			Tenchini et al., 1983
Estradiol 50-28-2	HSM	?	I [m]	hr	0.0007–0.7			Lycette et al., 1970
	HSC	?	−	hr		0.0007		
	HSF	?	I [n]	hr	0.0007–7			
Ethylene-thiourea 96-45-7	DON	y	−	hr, pp		3200		Teramoto et al., 1977

TABLE 1 (continued)

Chemical (CAS Registry No.)	Cells [a]	Tox [b]	Aneuploidy		Range tested [d] (μg/ml)	HIDT [e] (μg/ml)	LEDT [e] (μg/ml)	Reference
			+/−	Type [c]				
Griseofulvin 126-07-8	HPBL	?	+	ho, hr, pp, cm			40	Larizza et al., 1974
Mitomycin C 50-07-7	HPBL	y	−	ho, hr, hr:YY		1		Tenchini et al., 1983
MNNG 70-25-7	CHO	?y	+	hr, pp			hr: not given pp: 0.25	Bempong, 1979
	BPE	?	I [o]	ho, hr	0.1–5			Katoh et al., 1980
Mysoline (primidone) 125-33-7	HPBL	n	I [j]	ho, hr, pp	10–70			Bishun et al., 1975
Phenytoin 57-41-0	HPBL	n	I [j]	ho, hr, pp	10–70			Bishun et al., 1975
Rhodamine B 81-88-9	MF	y [k]	I [l]	ho, pp	2–20			Lewis et al., 1981
Testosterone 58-22-0	HSM	?	−	hr		0.7		Lycette et al., 1970
TPA (12-*O*-Tetradecanoylphorbol-13-acetate) 16561-29-8	MPE	?	+	hr			0.62	Dzarlieva and Fusenig, 1982
Vinblastine 865-21-4	CH	?	+	hr, pp, mp			0.05	Palyi, 1976
	DON	?	+	hr			~ 0.8	Hsu et al., 1983b
Zarontin (ethosuximide) 77-67-8	HPBL	n	I [j]	ho, hr, pp	10–70			Bishun et al., 1975

[a] Cell types: *Cells with limited lifespan*: HPBL, human peripheral blood lymphocytes — short-term culture; SHE, Syrian hamster embryo; BPE, bovine epithelium; RPE, rat tracheal epithelium; HSM, human synovial — male adult; HSC, human synovial — child; HSF, human synovial — female adult; MPE, mouse epidermis. *Continuous cell lines*: CHO, CHW, DON, V79, Chinese hamster continuous lines; HH, Hungarian hamster; MF, Muntjac fibroblasts.

[b] Tested to toxic levels; y, yes; n, no; ?, not given.

[c] Hr, hyperdiploid; ho, hypodiploid; pp, polyploid; mp, multipolar mitoses; cm, 'C-mitoses'; Hr:YY, hyperdiploidy specifically involving an extra Y chromosome; clones, aneuploid clones grown out; +, positive; −, negative; I, inconclusive.

[d] Shown for inconclusive results.

[e] HIDT, highest ineffective dose tested; LEDT, least effective dose tested.

Reasons for inconclusive results:

[f] Increase slight (4% cf. 0% in controls) and few cells scored (50 cells per point).

[g] Tested up to doses that increased the frequency of polyploidy 10-fold.

[h] Increase found in over 2000 cells per point, but lacks statistical significance and dose relation.

[i] No decrease in mitotic index.

[j] Probably negative but only 75 cells per point and not tested up to toxic dose levels.

[k] Compound caused structural chromosome aberrations.

[l] Increase was in hypoploid ($2n - 1$) cells and polyploidy. No hr reported.

[m] Increase ($P < 0.05$) only at lowest of 4 doses. No dose relation.

[n] Increase in 2 of 3 donors, but no dose relation: third donor had results at only 1 dose.

[o] Only slight increase in hr: effect is largely an increase in ho over weeks in culture.

TABLE 2

COMPOUNDS FOUND TO DISRUPT CELL DIVISION AND/OR INDUCE POLYPLOIDY (MUCH BASED ONLY ON QUALITATIVE DATA)

Chemical (CAS Registry No.)	Cells [a]	Mitotic effect (type) [b]	LEDT	Reference
Asbestos (crocidolite) 12001-28-4	SHE	Tetraploidy	2 $\mu g/cm^2$	Oshimura et al., 1984
	SHE	Anaphase lagging	1 $\mu g/cm^2$	Hesterberg and Barrett, 1985
Diazepam 439-14-5	JOK-1	C-Mitoses	50 $\mu g/ml$	Andersson et al., 1981
Diethylstilbestrol dipropionate 130-80-3	HSBP	C-Mitoses	15 $\mu g/ml$	Parry et al., 1982
	HSBP	Abnormal mitoses	10 $\mu g/ml$	Danford and Parry, 1982
Ethidium bromide 1239-45-8	DON	Multipolars, endos, abnor. centrioles	1 $\mu g/ml$	McGill et al., 1974, 1976
	TCH-2352	Endos	10–25 $\mu g/ml$	McGill et al., 1974
Gentian violet 584-62-9	CHO	Multipolars	1 $\mu g/ml$	Au et al., 1978
Griseofulvin 126-07-8	PtK_1	C-Mitoses	2.5×10^{-4} M	Mullins and Snyder, 1979
Halothane 151-67-7	V79	C-Mitoses, multipolars	0.5%	Sturrock and Nunn, 1976
Isopropyl (*N*-3-chlorophenyl) carbamate 101-21-3	3T3	Abnormal mitoses, ml*	10^{-4} M	Oliver et al., 1978
Joduron (3,5-diiodopyridon 4-*N*-acetic acid) 3737-08-4	HyCH	C-Mitoses	0.063%	Schmid and Bauchinger, 1976
Mercuric chloride ($HgCl_2$) 7487-94-7	HPBL	C-Mitoses	1×10^{-5} M	Verschaeve et al., 1984
Methylmercury (CH_3HgCl) 115-09-3	HPBL	C-Mitoses	5×10^{-6} M	Verschaeve et al., 1984
Metronidazole 443-48-1	V79	Polyploids, endos	10 mM	Korbelik and Horvat, 1980
Nitrous oxide 10024-97-2	HeLa	Multipolars, C-mitoses	80 lb/in^2	Brinkley and Rao, 1973
Nocodazole 31430-18-9	HeLa, CHO, W138, L(NCTC-929)	Mitotic arrest	0.04 $\mu g/ml$	Zieve et al., 1980
Olivetol 500-66-3	HPBL	Anaphase lag, unequal segr., multipolars	5×10^{-5} M	Morishima et al., 1976

TABLE 2 (continued)

Chemical (CAS Registry No.)	Cells [a]	Mitotic effect (type) [b]	LEDT	Reference
Potassium dichromate 7778-50-9	HEp-2	C-Mitoses, polyploidy	10^{-4} M	Majone, 1977
Sodium orthovanadate 13721-39-6	PtK_1	Chromosome movement inhibition		Cande and Wolniak, 1978

[a] Cell types: *Continuous cell lines*: HyCH, CHO, V79, DON, Chinese hamster; HeLa, HEp-2, human carcinoma; JOK_1, human leukemia; L(NCTC-929), 3T3, mouse; TCH-2352, cactus mouse; PtK_1, marsupsial kidney. *Cells with limited lifespan*: HSBP, W138 human fibroblasts; HPBL, human peripheral blood lymphocytes; PRE, rat tracheal epithelium.

[b] endo, endoreduplication; ml*, multilobed nuclei: microtubule and microfilament disruption seen by immunofluorescence.

Of these five, three are also chromosome-breaking agents (Table 3), i.e., TPA, DES and carbaryl. Benomyl has not been adequately tested for clastogenicity in mammalian cells although it is reportedly a plant cell clastogen (Zutsch and Kaul, 1975), and chrysotile asbestos is a weak clastogen (Oshimura et al., 1984).

The negative compounds are too few to draw any useful conclusions.

There are strong reservations about all the conclusions in this report because most compounds were tested only once, in one system and in one laboratory. Of the following 4 compounds that were tested more than once, colcemid was consistently positive, and the aneuploid clones established from colcemid-treated cells (Table 1) are conclusive proof that it induces aneuploidy in vitro. Vinblastine was positive in Chinese hamster cells in two laboratories. However, benomyl was positive in only one of two tests (Table 1) while MNNG was positive in one system but inconclusive in another.

(5) Discussion

(5.1) Possible cellular targets for aneuploidy induction

There are many mechanisms by which a chemical might induce aneuploidy. Recent investigations are elucidating the structure and function of the mitotic apparatus and in such studies lie not only fascinating cell biology but perhaps potential assay systems for compounds that might induce aneuploidy. The mitotic apparatus contains microtubules assembled from tubulin in the cytoplasm, in association with microtubule-organizing centers (MTOC) provided by centrosomes and, to a lesser extent, by kinetochores. The centrosome is a centriole surrounded by an amorphous cloud or halo required for its function; it has to be replicated each cell cycle for normal mitosis (see Mazia, 1984). The MTOCs are also known to contain nucleic acid which is thought to be important in their function (Pepper and Brinkley, 1980). Some of the effects that might lead to aneuploidy are:

— Alteration of microtubule assembly (e.g., by binding to or crystallizing tubulin, or through effects on enzymes or energy supply).
— Interference with centrosomes.
— Effects on kinetochores, such as interference with MTOC capability or with the timing and order of centromere separation.
— Persistence of nucleoli.
— Effects on genetic control of mitosis by mutation or by alteration at the transcription or translation levels.

These possibilities are discussed briefly below.

(5.1.1) Effects on the mitotic spindle and on microtubules

Several compounds are known to disrupt micro-

TABLE 3

SUMMARY OF RESULTS FOR IN VITRO ANEUPLOIDY ASSAY IN MAMMALIAN CELLS, WITH DATA ON STRUCTURAL ABERRATION INDUCTION

Aneuploidy conclusion	Compound	Structural aberration induction [a]			
		In vivo [b]	Ref.	In vitro	Ref.
Positive	Asbestos (chrysotile)	ND		W +	30
	Benomyl	–	1	I	2
	Benzo[*a*]pyrene	+ (MMN)	3–5	+ (+S9) [c]	6, 7
	Carbaryl	ND		I/+	8, 9/10
	Colcemid (colchicine)	I	11	W +	12
	Diazepam	ND		I (neg. –S9)	13
	Diethylstilbestrol	–/I (MMN)	4, 5/3	–/+	14/6
	Dimethylbenzanthracene	+	15	+	15
	Griseofulvin	–	16	I	17
	N-Methyl-*N'*-nitro-*N*-nitrosoguanidine	– (MSP)	15	+	15
	TPA	ND		+/I	18, 19/20, 21
	Vinblastine	ND		+	28
Negative	Cyproheptadine HCl [d]	ND		– [d]	27
	Ethylenethiourea	–	22, 23	–/I	22/7
	Mitomycin C	+	15	+	15
	Testosterone [d]	ND		ND	
Inconclusive	Benzanthracene	+	29	ND	
	Distamycin A	ND		ND	
	Estradiol	ND		ND	
	Mysoline	I	24	ND	
	Phenytoin	I	24	–	12
	Rhodamine B	ND		–/+/I	13/25/26
	Zarontin	ND		ND	

References: 1. Pilinskaya et al., 1980; 2. Gupta and Legator, 1976; 3. Salamone et al., 1981; 4. Kirkhart, 1981; 5. Tsuchimoto and Matter, 1981; 6. Dean, 1981; 7. Natarajan and van Kesteren-van Leeuwen, 1981; 8. Onfelt and Klasterska, 1983; 9. Ishidate et al., 1981; 10. Kazarnovskaya and Vasilos, 1977; 11. Sieber et al., 1978; 12. Galloway et al., in preparation; 13. Sasaki et al., 1980; 14. Tsutsui et al., 1983; 15. Reviewed by Preston et al., 1981; 16. Leonard et al., 1979; 17. Namba and Kimoto, 1976; 18. Emerit and Cerutti, 1981, 1982; 19. Dzarlieva and Fusenig, 1982; 20. Popescu et al., 1980; 21. Connell and Duncan, 1981; 22. Teramoto et al., 1977; 23. Shirasu et al., 1977; 24. Esser et al., 1981; 25. Lewis et al., 1981; 26. Au and Hsu, 1979; 27. Hite et al., 1977; 28. Segawa et al., 1979; 29. Peter et al., 1979; 30. Oshimura et al., 1984.

[a] From literature.

[b] –, negative; +, positive; I, inconclusive due to lack of dose relation, lack of statistical significance, lack of reproducibility or inadequate protocol; ND, no useful data located; MMN, mouse micronucleus test; MSP, mouse spermatocytes.

[c] +S9/–S9, with or without liver microsome activation system.

[d] Qualified negative, as not tested up to toxic dose levels.

TABLE 4

SUMMARY OF RESULTS FROM TABLE 3

Result in aneuploidy test in vitro (number of compounds)		Result in structural aberration test in vitro [a]			
		+	I	–	ND
Positive	12	8	4	0	0
Negative	4	1	1	1	1
Inconclusive	7	0	3	0	4

[a] +, + and +/I; I, I and +/– or I/–; –, –; ND, no useful data.

tubles, for example, podophyllotoxin (Horwitz et al., 1982), griseofulvin, which may act by inhibiting the combination of microtubules with microtubule-associated proteins (Roobol et al., 1976), colchicine and colcemid which inhibit tubulin polymerization (Margolis and Wilson, 1977), vinblastine which depolymerizes and crystallizes tubulin (Bensch and Malawista, 1969), methyl mercury, which disrupts tubulin by binding to sulfhydryl groups (Sager et al., 1983), and diazepam which appears to inhibit the separation of centrioles (Andersson et al., 1981). Although cells can under certain circumstances recover from the mitotic block produced by these compounds, incomplete recovery might lead to aneuploidy. Of interest is nocodazole which has colchicine-like effects, but differs in that cells reportedly recover more rapidly after removal of the compound (Zieve et al., 1980). This compound might be a useful model in development of in vitro aneuploidy tests. In contrast to these destabilizing compounds, taxol promotes assembly of microtubules in cells (Horwitz et al., 1982) although it abrogates the usual MTOCs.

There are several recent techniques for investigating microtubules in cells, for example, antitubulin immunofluorescence (Brinkley et al., 1975; Weber et al., 1975). In living cells the spindle is visible by polarized light microscopy. Tucker et al. (1977) found a close correlation between cytotoxicity of vinblastine and the frequency of cells in which the mitotic spindle had dissolved. Such direct observation of spindle disturbances could be useful in assessing aneuploidy inducers. Parry et al. (1982) stained cells to demonstrate both chromosomes and spindle fibers (fixation in the presence of Mg^{2+} and Ca^{2+} to maintain the spindle proteins, and staining with brilliant blue, for proteins, plus safranin O [red] for chromatin). Spindle fibers were absent in cells treated with colcemid, and observations on spindle behavior strengthened the authors' conclusion that diethylstilbestrol was a potential inducer of aneuploidy (Danford and Parry, 1982).

Before proposing microscopic studies of microtubule behavior as a technique for detecting aneuploidy inducers, a great deal more study is needed to elucidate the normal behavior of the cellular substructures. Also there are difficulties of interpretation; for example, not all microtubules stained by immunofluorescence disappear after colcemid treatment (Brooks and Richmond, 1983), and quiescent and cycling cells yield different results. Andersen and Ronne (1981, 1983) proposed that chromosome length measurement be used as an indication of spindle inhibition, reasoning that because spindle inhibitors increase the duration of metaphase, chromosome contraction would increase. Colchicine caused increased chromosome contraction (Andersen and Ronne, 1981) as did mercury and cadmium (Andersen and Ronne, 1983). The authors claim that this method might detect weak inducers of aneuploidy, although it is an indirect way to study the problem.

(5.1.2) Enzyme inhibition and effects on metabolism

A compound that was known to inhibit flagellar beating by inhibiting a dynein ATPase activity (*erythro*-9-[(3-(2-hydroxynonyl)]adenine; EHNA: Bouchard et al., 1981) also altered the spindle elongation thought to be important in anaphase in mammalian cells (Cande, 1982). This was taken to be evidence for a similar ATPase activity in mammalian cells. Since correct pH and concentrations of ATP, GTP and calcium ions are required for accurate microtubule assembly in cells (Deery and Brinkley, 1983), it is clear that compounds that alter many basic chemical states could affect mitosis.

(5.1.3) Microtubule-organizing centers (MTOC) and centrioles

Electron microscopy was used by Onishenko et al. (1979) to study centrioles in abnormal mitoses induced by 2-mercaptoethanol in various cell lines. At metaphase and telophase the number of centrioles at each pole of tri- or multipolar mitoses varied, and each centriole was surrounded by a halo whereas in a normal bipolar mitosis only one centriole at each pole had such a halo. The halo was believed to represent a structure necessary for the formation of a normal spindle. Based on the observation that normal cells contain two mature and two immature centrioles, it was postulated that the effects of mercaptoethanol were due to its induction of mitotic arrest; while the cells were held in mitosis, the second set of centrioles had time to mature and could therefore form spindles,

leading to multipolar mitoses on release of the block.

(5.1.4) Centromere separation

In mammalian cells centromere separation of the chromosomes is thought to take place in an ordered sequence (reviewed by Vig, 1984). It has been postulated that if a chromosome separates out of phase, it may fail to attach to the spindle fibers and nondisjunction may result (Vig, 1984). There is evidence for such nondisjunction of one of the X chromosomes in lymphocytes of females (Fitzgerald, 1975; Galloway and Buckton, 1978). Any compound that alters the time of separation and/or attachment to the mitotic apparatus might be a potential aneuploidy inducer.

(5.1.5) Persistence of nucleoli

The persistence of nucleoli during mitosis may affect the segregation of chromosomes, in particular those that bear the nucleolar organizers and are physically restrained by nucleolar material. Certain chemicals, including DNA synthesis inhibitors, increase the frequency of nucleolar persistence (reviewed by Heneen and Nichols, 1966) and effects of DNA viruses on nucleoli have been postulated as a mechanism of production of trisomy in man (Evans, 1967).

(5.1.6) Genetic control of mitosis

Mitosis is under multi-gene control, and mutants could be useful in studying mitosis and in assaying compounds that might induce aneuploidy, much as cell proliferation mutants have been used (e.g., Baserga, 1984). Some examples of known mutants are temperature-sensitive mutants in α- and β-tubulin (Cabral et al., 1980, 1981), and cells resistant to colcemid binding (Ling et al., 1979) or griseofulvin (Cabral et al., 1980). There are also mutants resistant to taxol which actually require taxol for mitosis (Cabral, 1983), and mutants of microtubule-associated proteins (Gupta and Gupta, 1984).

(5.1.7) Damage to chromatin and nucleic acids

It is interesting that many of the compounds apparently positive for induction of aneuploidy in vitro are also clastogens (Table 3). Could hyperdiploidy seen in culture result from the clastogenicity of any of these positive compounds? Since the experimental design in vitro used a short period (usually a few cell cycles at most) and tested for whole chromosome gain, it is unlikely. Clastogens might cause meiotic aneuploidy by induction of chromosomal rearrangements that interfere with pairing at meiosis but this mechanism does not apply in vitro. However, since clastogens can affect DNA structure or function they might influence genes that control accurate chromosome segregation at mitosis. There is also evidence for nucleic acids integral to kinetochores (especially DNA) and centrosomes (especially RNA; Pepper and Brinkley, 1980). If this is important for MTOC function, the mitotic process could be altered by compounds such as alkylating agents that bind covalently to nucleic acids. Also, alkylators could bind to nucleoside triphosphates such as ATP and GTP, indirectly affecting many processes required for mitosis. Alkylation of proteins is a further possibly that should not be ignored. It is more difficult to explain the apparent clastogenicity of vinblastine sulfate and of colchicine (Table 3). These compounds are not thought to interact directly with DNA, but could cause physical breakage during mitosis by affecting persistence of nucleoli (e.g., Heneen and Nichols, 1966), and chromosome stickiness.

(5.2) Possible areas of future development in aneuploidy detection

(5.2.1) Automated analysis

In common with tests for structural aberration induction in vitro, a limitation on counting metaphase chromosomes is the time it takes to count a large enough sample of cells for meaningful statistical analysis.

To alleviate the problem of laborious microscope work, an automated system for chromosome counting would be useful. Some such systems are in use or under development (reviewed by Shafer et al., 1985) for finding and analyzing metaphase cells in conventional preparations on microscope slides. There are also examples of application of DNA measurement by flow cytometry to aneuploidy assessment (Vanderlaan et al., 1983). The system measures DNA content of individual nuclei by detecting DNA-bound fluorochrome. The

method can clearly demonstrate the presence of cells with even multiples of the haploid DNA content (e.g., 3C or 4C cells) but such polyploid cells may not necessarily be associated with aneuploidy induction. In comparisons of the variation in DNA content at G_1, difficulties arise in interpreting a spread around the peak of normal DNA content, because losses or gains of DNA could result from structural chromosome aberrations. Indeed, the same system has been applied to detection of clastogens (e.g., Otto et al., 1984). In conjunction with conventional metaphase or anaphase analysis to check that breakage does not account for the altered DNA content, flow measurements might be useful for studying large numbers of cells, giving good statistical resolution, but may not be able to detect weak effects because of the intrinsic variability in the system.

(5.2.2) Anaphase analysis and micronuclei

A general study of aberrations at anaphase is not helpful in detecting aneuploidy. However, if metaphase analysis shows no evidence for breaks and fragments, anaphase laggards may be taken as evidence for potential whole-chromosome aneuploidy. Similarly, micronucleus formation by non-clastogens would suggest nondisjunction. Techniques for identifying centromeres or associated material (e.g., C banding; silver staining of 'centromeric dots'; immunofluorescent staining of kinetochores) might usefully be applied both to micronuclei and to anaphase laggards. The presence of a centromere would increase the likelihood that the lagging material or micronucleus contained a whole chromosome. Detection of the centromere-containing body may be amenable to flow cytometry or other automated methods of detection.

(5.2.3) Restriction fragment length polymorphisms (RFLPs)

There is recent molecular evidence that non-disjunction may play a part in development of certain tumors. Individual chromosomes may now be identified by the pattern of DNA fragment lengths obtained after digestion with restriction enzymes, because these patterns vary from homologue to homologue (restriction fragment length polymorphisms). Analyses of RFLPs have suggested that in some cases of retinoblastoma, for example, two copies of the same homologue exist in the tumor; it is thought that the chromosome carrying a recessive gene associated with oncogenesis may be duplicated and the wild-type chromosome lost (Dryja et al., 1984; Cavenee et al., 1983). It remains necessary to demonstrate that the observations are not the result of mitotic recombination in the region between the centromere and the RFLP markers used. The methods for RFLP analysis are not yet routinely used in many labs and entail much detailed work, but it is possible that similar techniques might be useful to investigate aneuploidy induction in vitro.

(6) Testing recommendations

Clearly, the amount of data on potential aneuploidy inducers is sparse and no definitive protocol for in vitro screening in mammalian cells exists. A research effort is required to develop good test systems, but some general recommendations can be made for protocol requirements.

(6.1) Cell source

We recommend the use of diploid cells for testing. Because established cell lines are often heteroploid, primary or early passage cells are desirable. If continuous cell lines are used they should be clones with very little variability around the modal number. There are at least two reasons for this requirement: if the background level of variability is high, statistical resolution of small differences is difficult, and the inherent stability of heteroploid cells may be altered so that the meaning of an increase in aneuploidy is questionable. Also polyploid cell lines may give misleading results because loss and gain of chromosomes may be more compatible with cell survival than in diploid cells. Examples of studies we considered unsuitable because of the background variability are a report on Chinese hamster fibroblasts (CH1-L) cells (Danford, 1984) in which about 30% of the cells were non-modal despite the early passage number, and a study on Ehrlich ascites cells described as having 36% hypodiploid cells (less than 40 chromosomes), 48% in the 41–60 chromosome range, and 16% hypertriploid or tetraploid cells

(Bishun and Pentecost, 1981). A suitable background rate might be 0–5% cells with $(2n + 1)$ chromosomes, since hyperdiploidy in primary cells is rare. In one study on human lymphocytes, pooled data on 48-h cultures from 280 donors showed that of 31 773 cells, 0.1% had 47 or 48 chromosomes, and 7.9% had 44 or 45 chromosomes (Brown et al., 1983).

(6.2) Types of abnormality

Hypodiploidy alone is not usually acceptable evidence for aneuploidy induction, because of the large contribution of artefacts (e.g., in slide preparation) to the total amount of hypodiploidy observed and because of the potential confounding effect of membrane fragility induced by the test compound. Observations of hypodiploidy should still however be recorded separately. Chromosome numbers should be recorded and data presented to show frequencies of cells with each number and of polyploid cells or other observations. Hyperdiploidy is generally the acceptable evidence for aneuploidy, except in cases where monosomic clones are cultured through several divisions and identified as such.

(6.3) Controls

Adequate concurrent negative and/or solvent controls are required. Since there is no well-established positive control, and the potential modes of aneuploidy induction are many, use of a positive control cannot be made an acceptance criterion for an experiment. Use of colchicine or colcemid may however yield useful information about the system under investigation, and an attempt should be made to identify and use positive controls.

(6.4) Dose selection

The dose levels should include a dose that produces some evidence of cytotoxicity such as cell death, mitotic inhibition, or even structural aberrations in an attempt to show that the compound entered the cells. However, it is important to include non-toxic doses in the assay, and to take toxicity into account in interpretation of results because of possible non-specific effects of cytotoxicity. A wide range of doses (several orders of magnitude) should be tested initially to avoid missing any active range. A more closely spaced series of doses may then be selected for aneuploidy testing. Careful attempts should be made to establish the shape of the dose–response relation because of the multiplicity of possible types of targets in the cell and the possibility that threshold levels may exist when certain mechanisms are involved.

(6.5) Number of dose levels

Since both a toxic and a not-demonstrably-toxic dose should be used, at least two doses are required and more are desirable not only to establish a dose relation if possible, but to avoid missing activity that occurs over a very narrow concentration range, for example at doses just below limiting toxicity.

(6.6) Fixation time

Because the end point is numerical change arising from unequal segregation, induction of aneuploidy cannot be established by counting chromosomes in metaphases of the first mitosis after treatment. In contrast, tests for structural aberrations should use the first post-treatment metaphase, so that the best protocols for structural and numerical aberrations are mutually exclusive. The cell cycle length must be established and the protocol designed to examine cells in their second or subsequent metaphase after treatment, making allowance for any test-compound-induced delay of cell cycle progression. In cases where the effects found are mitotic anomalies such as C-mitotic effects or multipolar mitoses, the first post-treatment metaphases may be used, but these end points are not acceptable evidence for aneuploidy; they suggest a need for further investigation.

(6.7) Number of cells scored

In determining the numbers of cells to be scored the investigator should consider the level of detection possible at a given sample size. Because tester cells should have a low rate of hyperdiploidy, large cell samples are needed. This is exemplified by a study in which metaphase cells with two Y chro-

mosomes were counted (Tenchini et al., 1983). Even with a large sample of cells scored (1000–2000 per dose level) it was not possible to demonstrate statistical significance of the increase induced by colcemid. Statistical methods for frequency analysis of hyperdiploidy are needed.

(6.8) Metabolic activation

In vitro activation systems were used rarely in the literature we reviewed. Many primary and early passage cells have some intrinsic activation capacity: this should be characterized if possible, (e.g., by demonstrating SCE induction or toxicity by indirect mutagens such as cyclophosphamide or benzo[*a*]pyrene). Otherwise consideration should be given to use of microsomal preparations such as liver homogenates, or of feeder layers of metabolizing cells.

(6.9) Physical conditions

The temperature and pH in the experiments should be carefully controlled or at least measured. There is evidence that pH can affect aneuploidy observations in lymphocytes (Shimada and Ingalls, 1975). Temperature fluctuations also interfere with mitosis in cultured cells (Rao and Engelberg, 1966), although rather large temperature shifts (e.g., from 37°C to 29°C) were used to demonstrate this effect. An overall recommendation is to maintain the cultures in conditions as close as possible to the physiological environment. It seems prudent to control, for example, osmotic strength of the medium with test compound, because ionic strength is crucial to many cellular processes. Increases in osmolality have been shown to affect genetic end points in cultured cells such as chromosome structural aberrations (Galloway et al., 1985).

(7) Conclusions

In test development, emphasis should not only be on good protocol design, but on possible methods of validation. The goal would be to see whether effects seen at metaphase a few cell cycles after treatment persisted in the form of true aneuploid cells, or could be demonstrated also in whole animal systems.

The development of in vitro tests for aneuploidy induction is an area in need of much research effort. The multiplicity of potential mechanisms of interference with normal control of chromosome segregation makes this a complicated but fascinating challenge.

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